

Review

Role of the Apoptotic and Mitotic Regulator Survivin in Melanoma

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Abstract. *Melanoma is the deadliest form of skin cancer. Melanoma develops in response to genetic and environmental pressures which lead to oncogenic transformation of normal human melanocytes, the pigment-producing cells in the body. The majority of melanoma-associated deaths are due to metastases, highlighting the importance of understanding the molecular mechanisms driving melanoma development and progression. This review focuses on survivin, and its involvement in the melanoma biology. Since its identification in the late 1990s, a vast body of work has been generated, demonstrating the role of survivin in various malignancies. This review discusses the established mitotic and cytoprotective properties of survivin, and its potential role in melanoma development and progression. A newly recognized functional property of survivin is also discussed, namely enhancement of cellular motility, which may underlie its role in promoting melanoma metastasis. Finally, various therapeutic strategies targeting survivin in melanoma are reviewed.*

Melanoma incidence has been increasing steadily over the past two decades, with over 60,000 new cases of invasive disease diagnosed each year in the United States alone (1). When detected early, treatment by surgical excision is usually curative; however, survival is poor for patients with metastatic disease. Most melanomas occur sporadically, with familial melanoma accounting for fewer than 10% of cases (2). Genome-wide association studies have identified chromosome 9p21 as harboring the most commonly altered gene in familial melanoma (2). This locus contains the cyclin-dependent kinase

inhibitor 2A (CDKN2A) gene, which encodes both p16 and p19^{ARF} tumor suppressors (3). The most common genetic risk factors for melanoma are associated with pigmentation phenotypes, such as fair skin, blond or red hair and blue eyes, as well as numerous melanocytic nevi (moles) (4). The predominant environmental factor promoting melanoma development is exposure to DNA-damaging ultraviolet (UV) light, primarily from the sun or tanning beds.

Melanoma Metastasis and New Targeted Therapies

Primary melanoma has a very high cure rate if detected early. Following metastasis, however, it becomes increasingly less likely that patients will survive their disease (5). Metastasis describes a process by which tumor cells migrate away from the primary tumor site, are transported through the circulatory or lymphatic system, and then adhere to and invade new sites where they establish viable tumors (6). Thus the ability of tumor cells to migrate and to invade is essential for successful metastasis.

Therapeutic advances have been made over the past few years, leading to Food and Drug Administration approval of two agents aimed at metastatic melanoma. The first agent is the serine/threonine protein kinase B-Raf (BRAF) inhibitor PLX4032 (vemurafenib) (7), which targets the BRAF V600E mutation present in 60% of all melanomas (8), leading to inactivation of the mitogen-activated protein kinase (MAPK) pathway. Early clinical trials have demonstrated an 80% initial response rate among patients with V600E-positive melanoma, although most patients experience relapse (7) due to acquired resistance to BRAF inhibition. Acquired resistance usually does not involve additional BRAF mutations, but rather up-regulated signaling through activated N-Ras (NRAS) (9), overexpression of platelet-derived growth factor B (9), or protein kinase COT-dependent reactivation of extracellular signal-regulated kinases (Erk) (10).

A second agent, ipilimumab, is an antibody against cytotoxic T-lymphocyte antigen 4 (CTLA-4) and has also

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been approved for metastatic melanoma treatment. In a phase III clinical trial, patients treated with ipilimumab had a median overall survival of 10 months in comparison to 6.4 months for control patients (11). Unfortunately, because it works by hyperactivating the immune system, ipilimumab may trigger severe autoimmune side-effects (12). The overall response rate is 11% (12), and at present it is not possible to predict which patients will respond to ipilimumab. A better understanding of the molecular biology of melanoma may eventually increase our capacity to select which patients may be most responsive to particular therapies.

Structure and Subcellular Localization of Survivin

First cloned in 1997, survivin is the smallest member of the inhibitor of apoptosis protein (IAP) family (13). The *survivin* gene spans approximately 14.7 kb on chromosome 17, and its transcription is initiated from a GC-rich promoter that lacks a Goldberg-Hogness (TATA) box. Transcription generates full-length (wild-type) survivin, along with four other splice variants (14). Unlike other members of the IAP family, survivin has a single baculoviral IAP repeat (BIR) domain and also lacks the really interesting new gene (RING)-finger domain characteristic of other IAPs (15). Additionally, survivin lacks a caspase-associated recruiting domain (CARD), which is critical for other IAP family members to bind and inactivate caspases, the effectors of apoptosis (16). Survivin is also distinguished from other IAPs because of its bi-functionality as a regulator of both apoptosis and mitosis (17), as discussed below.

Expression of survivin is developmentally regulated as it is ubiquitously expressed in fetal tissues, silent in most adult tissues, and then re-expressed in most types of cancers (18). More recent studies using sensitive detection methods have demonstrated survivin expression in some normal adult tissues. These include vascular endothelial cells (19), and developing hematopoietic and immune cells (20-22). Although there are five distinct isoforms of survivin, the predominant and most studied is the wild-type full-length survivin. There has been speculation that the different cellular functions may be attributed to different splice variants but this has been difficult to determine given the low level of expression of the minority variants (23). Only wild-type survivin has actually been investigated in melanoma (discussed below).

There are distinct subcellular pools of survivin located in the cytosol, mitochondria, and nucleus (24, 25). These subcellular pools are believed to be strongly tied to its varying cellular functions (Figure 1). Studies have demonstrated that the nuclear pool mediates survivin function in mitosis, while the cytosolic and mitochondrial fractions are responsible for its antiapoptotic function (25-27). Survivin shuttles from the nucleus to the cytoplasm

under the control of an evolutionarily conserved exportin-1 (Crm-1) nuclear export signal (NES). When this NES is mutated, survivin is trapped in the nucleus, proper cell division does not occur and the antiapoptotic function of survivin is also lost (28).

In tumor cells, survivin localizes to the inner mitochondrial membrane (25). In response to apoptotic stimuli, survivin is trafficked from the mitochondria to the cytosol where it can inhibit apoptosis (25). It has been shown that phosphorylation of survivin on residue Ser²⁰ by protein kinase A occurs in the cytosol but not in the mitochondria, and this differential phosphorylation regulates tumor cell apoptosis by modulating the interaction of survivin with X-linked inhibitor of apoptosis protein (XIAP) (29).

Survivin Expression and Its Role as a Prognostic Factor in Melanoma

The expression of survivin at each stage of melanomagenesis has been characterized. Survivin is absent from normal melanocytes, but we have shown it to be expressed in a broad spectrum of human melanomas (including localized and metastatic) (30) and melanocytic nevi including dysplastic, neurotized intradermal, and Spitz nevi (30, 31). In dysplastic nevi, survivin is primarily localized to the cytosol, although some nevi may exhibit nuclear survivin (32, 33). A more recent study revealed positive nuclear immunoreactivity for survivin in a large subset of melanomas but much less frequently in common and dysplastic nevi (34). In addition, nuclear survivin immunoreactivity was significantly less common in acral *versus* other melanoma types, in which nuclear survivin staining significantly correlated with poor survival (34, 35).

We have shown that survivin expression is also induced upon melanocyte transformation or in response to disruption of the tumor suppressor genes *TP53* (p53) or *retinoblastoma* (*Rb*) (36). Molecular profiling has identified survivin as a marker of poor prognosis and as an indicator of treatment resistance (37). Additionally, several studies have indicated that survivin can serve as a biomarker for a number of malignancies (38-40). With respect to melanoma, survivin expression in sentinel lymph nodes of patients with melanoma is inversely correlated with progression and mortality, as 61.5% of stage III patients who exhibited survivin-positive sentinel nodes (by reverse transcription-polymerase chain reaction, RT-PCR) died from their disease (41). On the other hand, all those with survivin-negative sentinel nodes were still alive after the 5-year observation period (41). Survivin levels may also be predictive of treatment outcome in melanoma, as one study reported that in patients with recurrent metastatic melanoma, lower levels of survivin are associated with significantly improved survival of those who receive postoperative adjuvant immunotherapy (42).

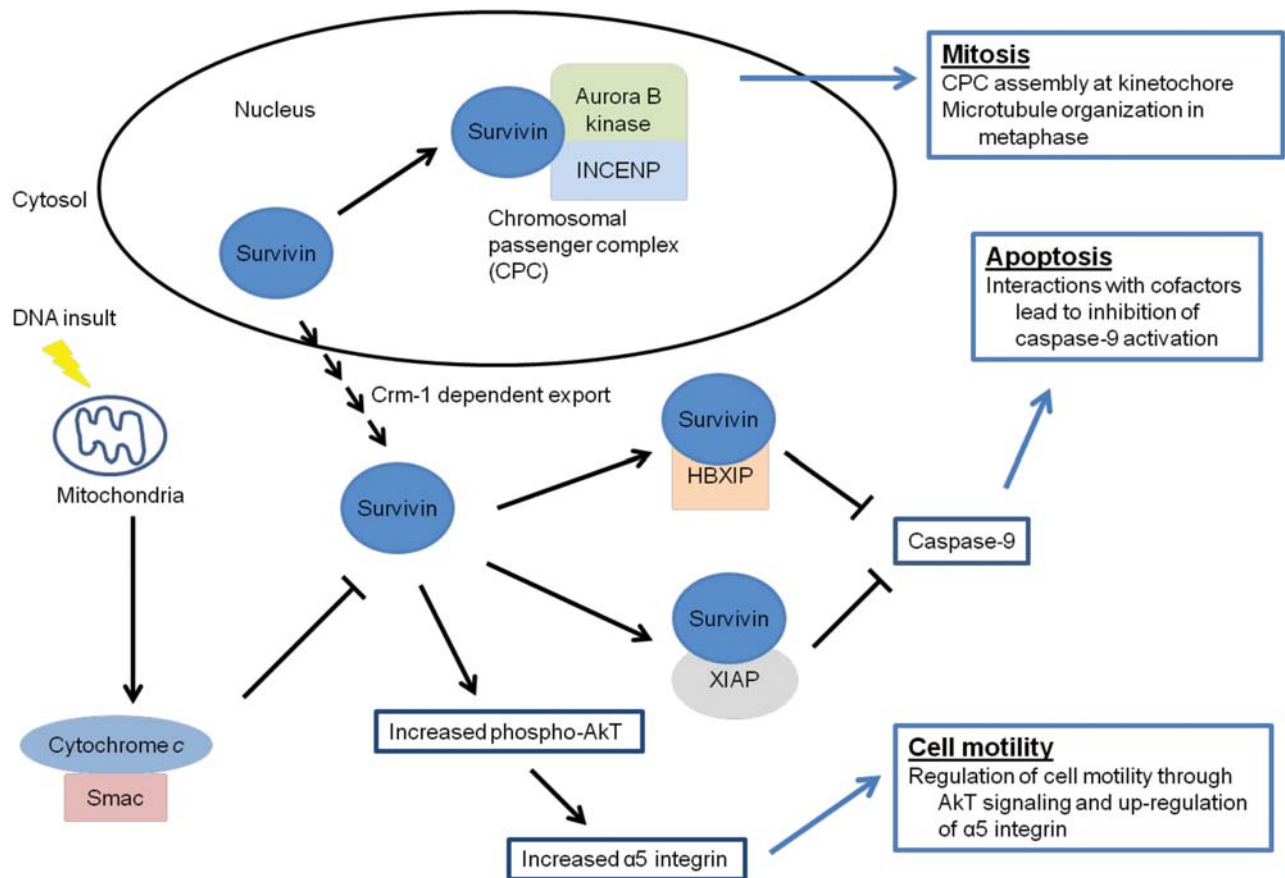


Figure 1. Cellular functions of survivin that contribute to tumor development and metastasis. Survivin is a multifunctional protein whose involvement in regulating mitosis, apoptosis, and cell motility can provide proliferative and metastatic advantages to tumor cells. Survivin forms part of the chromosomal passenger complex (CPC) with aurora B kinase, and the inner centromere protein (INCENP) to regulate chromosomal alignment during mitosis. Survivin interaction with hepatitis B X-linked interacting protein (HBXIP) and X-linked inhibitor of apoptosis protein (XIAP) prevent the activation of caspase-9, an effector molecule of programmed cell death. The anti-apoptotic function of survivin can be inhibited by the pro-apoptotic molecule Smac, which is released from mitochondria when the intrinsic apoptotic pathway is triggered. Survivin can also promote melanoma cell motility through activation of AKT and up-regulation of $\alpha 5$ integrin.

Regulation of Survivin Expression and Post-translational Modifications

Numerous studies have identified various genetic elements which exert transcriptional and translational control over survivin expression. Basal *survivin* gene expression is mediated through binding of specificity protein-1 (SP-1) to the GC-rich region of the survivin promoter (43). A role for p53 in suppressing *survivin* expression has been shown (44), and our more recent work has demonstrated that knockdown of either p53 or Rb protein in melanocytes is sufficient for survivin induction (36). Although the E2F transcription factor E2F2 has been previously characterized as a positive regulator of transcription, we identified a novel functional E2F2-binding site in the *survivin* promoter and found that E2F2 acts downstream of Rb to function as a negative

regulator of survivin (36). We also found that mutation of either the p53- or E2F2-binding sites in the *survivin* promoter increased transcription (36). These findings suggest that perturbations in the p53 or Rb pathways, which may occur as a result of loss of alternative reading frame (Arf) or p16 proteins in melanoma (3), can result in up-regulation of survivin expression.

There may also be a role for other tumor suppressors in the regulation of survivin expression in melanoma. Caveolin-1 inhibits *survivin* gene transcription by preventing lymphoid enhancer factor (Lef)-promoter binding in an E-cadherin-dependent manner, and this mechanism is operative in mouse melanoma (B16-F10) cells, leading to increased apoptosis (45). Thus loss of E-cadherin expression, which frequently occurs in melanoma, may result in reduced activity of caveolin-1 and consequent up-regulation of survivin

expression. Epigenetic modifications may also be important in regulating survivin expression, as histone deacetylation can direct methylation and silencing of the *survivin* promoter (46), although this pathway has not been investigated in melanoma.

Several post-translational modifications have been described which regulate survivin stability and function, namely phosphorylation and ubiquitination. Cyclin-dependent kinase 1 (CDK1)-mediated phosphorylation of Thr³⁴ is vital for survivin anti-apoptotic function (47), and we have shown that a non-phosphoryl Thr³⁴Ala mutant blocks growth of human melanoma tumor xenografts (48, 49). Survivin is also phosphorylated at Ser²⁰ by polo-like kinase 1 (50) and at Thr¹¹⁷ by aurora B kinase (51); while these modifications are important for mitotic regulation by survivin, their role in melanoma has not been investigated. Survivin is highly expressed in the G₂M phase of the cell cycle, and degraded *via* the ubiquitin-proteasome pathway during G₁ (52). In various cell types, epidermal growth factor (EGF) (53) and cyclooxygenase-2 (54) signaling inhibit ubiquitin-mediated degradation of survivin, leading to increased apoptotic resistance. On the other hand, XIAP-associated factor 1 (XAF1) is a putative tumor suppressor that can reverse the antiapoptotic activities of survivin by targeting it for ubiquitination (55). Finally, interaction of survivin with the chaperone protein heat shock protein (Hsp) Hsp90 has been shown to increase survivin stability and threshold for apoptotic stress in cancer cells (52).

Survivin Function in Cell Division

Disruption of survivin function, at least in malignant cells, results in cell cycle defects including multipolar mitotic spindles, failure of cytokinesis, and formation of multinucleated cells (56). We have shown that expression of a dominant-negative survivin mutant in melanoma cells results in loss of G₂/M DNA content and in reduced proliferation both *in vitro* (30) and *in vivo* (48). Survivin is a chromosomal passenger protein (57) that interacts with other passenger proteins including aurora B kinase and inner centromere protein (INCENP) (58) to facilitate movement of the chromosomal passenger protein complex from the inner centromere during prometaphase to the midbody during cytokinesis (59). Survivin is also involved in microtubule spindle assembly and organization (60). It is plausible that survivin may help tumor cells that have sustained DNA damage to bypass cell cycle checkpoints and proceed with cell division.

Survivin Function in Apoptosis

Consistent with its unique protein structure compared to other IAPs, survivin exerts its anti-apoptotic function in a

different manner (Figure 1). While conventional IAPs such as XIAP, livin, and cIAP1/cIAP2 directly bind to procaspases, ubiquitinate them, or prevent their proteolytic cleavage and subsequent activation (61), survivin does not appear to directly bind to caspases. Rather, survivin exerts antiapoptotic control by binding to and stabilizing XIAP, which inhibits caspase-9 (62). Survivin-mediated inhibition of caspase-9 has also been shown to be dependent on binding to a co-factor hepatitis B X-interacting protein (HBXIP) (63). The antiapoptotic function of survivin is negatively regulated by release of second mitochondrial-derived activator of caspase (Smac) from mitochondria, upon triggering the intrinsic cell death pathway (64).

We have characterized the antiapoptotic role of survivin in normal human melanocytes and in human melanoma cells. We have previously demonstrated that forced expression of survivin blocks both caspase-dependent and -independent cell death in human melanocytes (65). In melanoma cells, we have shown that survivin can protect against caspase-independent apoptosis (66). Numerous studies have demonstrated the pro-apoptotic activity of dominant-negative survivin mutants in melanoma cells which increase the sensitivity to cytotoxic drugs *in vitro* (30), and reduce melanoma tumor growth *in vivo* (48, 49). Using a mouse model with melanocyte-specific expression of survivin, we also demonstrated that survivin not only confers protection against apoptosis, but also promotes the development of UV-induced melanoma and tumor metastasis to lymph nodes (67).

A Novel Role for Survivin: Promotion of Cellular Motility

Cell motility encompasses both migration and invasion, which are key aspects of the metastatic process (6). Both migration and invasion require signaling events within the cell and with the extracellular matrix for navigation of tumor cells within their microenvironment and to distant sites. Recent studies have implicated survivin in these processes, which may underlie its role in promoting cancer metastasis. Mehrotra *et al.* (68), using the breast adenocarcinoma line MDA-MB-231, showed that survivin co-operatively binds XIAP and mediates both cell invasion, as well as metastasis *in vivo*, independently of its antiapoptotic function. Survivin-mediated invasion was integrin-independent and required activation of nuclear factor (NF)- κ B and cell motility kinases focal adhesion kinase (FAK) and sarcoma-related (Src) kinase (68).

We have also recently shown that survivin enhances cell migration and invasion of human melanoma cells (69). In our system, promotion of motility by survivin was found to be dependent on activation of the protein kinase-B (Akt) signaling pathway and upregulation of α 5 integrin (which both occur following survivin expression in melanocytes or its overexpression in melanoma cells), as blocking either of

these molecules abrogated survivin-enhanced migration (69). Knockdown of survivin by RNAi, under conditions where apoptosis was not induced, further demonstrated that survivin is required for constitutive migration and invasion of melanoma cells (69). An additional finding was that survivin-mediated promotion of melanoma cell invasion is also dependent on activation of the MAPK pathway, as evidenced by blocking with inhibitors of Erk phosphorylation (69). By contrast, overexpression of survivin is not consistently associated with Akt activation or $\alpha 5$ integrin up-regulation in other (non-melanoma) cell lines (J. McKenzie, T. Liu and D. Grossman, unpublished data).

Targeting Survivin in Melanoma

There are two main factors that give credence to survivin as a therapeutic target in cancer. Firstly, its minimal expression in most normal tissues compared to robust expression in most cancer cells may provide a broad therapeutic window through which tumors may be treated without detrimental side-effects on normal cells. Secondly, survivin appears to be critically involved in multiple signaling pathways that promote tumorigenesis, which suggests that inhibition of survivin may allow targeting of multiple mechanisms underlying tumor growth and metastasis. It is important to recognize that survivin is not a 'traditional' drug target in that it is not a cell surface protein that can be targeted by antibodies, and does not possess a catalytic domain which is usually the target of small molecule inhibitors (70).

Here we provide a synopsis of a number of promising therapeutic strategies targeting survivin, and their current state of clinical development. Several agents are being tested on patients in early clinical trials (70). These strategies are all geared to reduce tumor growth and induce an apoptotic response in malignant cells. While some of these approaches have not been examined specifically in melanoma, they may be applicable to melanoma, as well as to other tumor types.

Antisense oligonucleotides. Developed by Eli Lilly and Co., LY2181308 is an antisense oligonucleotide molecule designed to target *survivin* mRNA and inhibit its expression in tumor cells. LY2181308 binds to the translation initiation codon on *survivin* mRNA, blocking translation and leading to degradation of the transcript (71). Survivin levels are reduced by LY218308, leading to inhibition of growth of colorectal cancer cells both *in vitro* and in mouse models (72). In a dose-escalation study, patients were administered LY218308 intravenously before and after tumor biopsies to demonstrate reduction in survivin protein expression and restoration of apoptotic signaling *in vivo* (73). Patients are currently being recruited for a phase II study examining the effect of LY2181308 in combination with docetaxel in non-small cell lung cancer (ClinicalTrials.gov number, NCT01107444).

Potential pitfalls associated with the use of oligonucleotides include limited stability *in vivo* and inefficient targeting and neutralization of *survivin* mRNA (70).

Hammerhead ribozymes. Ribozymes have been developed as an alternative to oligonucleotide antisense molecules. Ribozymes are small RNA molecules with specific endonucleolytic activity that catalyze the breakage of phosphodiester bonds, resulting in cleavage of RNA substrates (70). Expression of anti-survivin ribozymes in melanoma cells resulted in reduced survivin expression and an increased apoptotic response to radiation (74) and *cis*-platinum (75). The utility of such ribozymes, however, may be limited by potential problems with degradation and aberrant cell trafficking (70).

Transcriptional repression. YM155 is a small molecule inhibitor that suppresses transcription of *survivin* by binding to its promoter. There have been a number of *in vitro* and pre-clinical studies conducted to evaluate the pro-apoptotic activity of YM155 in a variety of human cancer lines. The drug effectively suppressed growth of prostate cancer xenografts (76), and more recent studies have demonstrated broad apoptotic activity against numerous cell lines and xenografts including melanoma (77).

A recently concluded phase II study found that YM155 was modestly effective at suppressing tumor growth in patients with refractory advanced non-small cell lung cancer (78). However, a multicenter phase II trial of YM155 in patients with unresectable stage III or IV melanoma concluded that YM155 is not effective as a single agent, as only one out of 29 patients showed a partial response (79). Although other studies have documented the predictive value of survivin levels (41, 42), a significant caveat of this and other studies is that the expression levels of survivin in tumors pre- and post-therapy was not examined.

Survivin vaccines and immunotherapies. The main focus of cancer vaccines and immunotherapies is on tumor-associated antigen recognition by cytotoxic T-cells. Several phase I and II studies are being conducted to evaluate the efficacy of survivin vaccines. One study (ClinicalTrials.gov number, NCT00108875) is examining the immunological response and clinical outcome to vaccination with survivin peptides in patients with advanced melanoma, pancreatic, colon, and cervical cancers. Another study (ClinicalTrials.gov number, NCT00573495) is recruiting patients with advanced breast cancer for a phase I study to examine activation of the immune system in response to survivin and telomerase peptides.

Indirect anti-survivin therapies. In addition to targeting survivin directly, other strategies are being employed to target many of the signaling molecules downstream of

survivin. For example, preclinical studies have been performed with sherdin, which is an HSP90 peptidomimetic. Sherdin inhibits the survivin-HSP90 interaction by occupying the ATP pocket of HSP90, destabilizing its interaction with survivin leading to induction of both apoptotic and non-apoptotic cell death (80). Sherdin has only been tested in preclinical tumor models, and has yet to make the transition to human clinical trials. Additionally, other small molecule inhibitors targeting CDK1 and TCF are being evaluated in phase I/II studies (37).

Conclusion

In summary, survivin is an essential component of numerous cancer networks. Its diverse functions in cell division, apoptosis, and motility are all implicated in melanoma tumor development and metastasis (Figure 1). Survivin function is critically regulated by a number of signaling pathways. Although therapeutic targeting of survivin has shown some promise, the efficacy of such strategies may be augmented if used in combination with other therapies. Such an example might be combining a BRAF inhibitor with a survivin-targeted therapy in patients with melanoma. Further investigation into the biology of this nodal protein is warranted and it remains to be seen how targeting survivin will be truly beneficial for cancer patients. These studies have highlighted a potential new area, where melanoma treatment strategies can be developed by targeting IAPs.

Conflicts of Interest

The Authors declare no conflicts of interest.

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